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14. ABSTRACT Zinc deficiency is surprisingly common in the US. One organ expected to suffer during zinc deficiency is the prostate, since it is the greatest tissue accumulator of zinc in men. Zinc uptake in prostate epithelial cells is higher than most other cells types in the body, but zinc levels in these cells rapidly decrease upon transformation to a cancerous phenotype. Zinc homeostasis may play important regulatory roles in both normal growth and carcinogenesis within the prostate, however the mechanism remains obscured. The cytoskeleton is a known target of zinc binding in the cell, especially in microtubules. Crystallization studies have defined binding sites for zinc in microtubules, but cellular evidence is lacking. However, it has been proposed that microtubule dynamics may be disrupted when cellular zinc levels drop. Current drugs approved for use or in clinical trials to treat prostate cancer (paclitaxel, docetaxel, and 2-methoxyestradiol) target the microtubule network resulting in cell death. It is unknown if zinc deficient individuals have alterations in the microtubule network such that the activity of these chemotherapeutic agents is altered. Therefore, we tested the hypothesis that inadequate amounts of zinc disrupts microtubule function and decreases efficacy of microtubule-targeting chemotherapeutic agents in prostate cancer cells.					
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INTRODUCTION

Zinc deficiency is surprisingly common in the US [Wakimoto and Block, 2001]. One organ expected to suffer during zinc deficiency is the prostate, since it is by far the greatest tissue accumulator of zinc in men [Boyle *et al.*, 2003]. Zinc uptake in prostate epithelial cells is higher than most other cells types in the body. Interestingly, zinc levels in these cells rapidly decrease upon transformation to a cancerous phenotype [Costello and Franklin, 1998]. Zinc homeostasis may play important regulatory roles in both normal growth and carcinogenesis within the prostate, however the mechanism remains obscured. The cytoskeleton is a known target of zinc binding in the cell, especially in microtubules. Crystallization studies have defined binding sites for zinc in microtubules, but *in cyto* evidence is lacking [Löwe *et al.*, 2001]. However, it has been proposed that microtubule dynamics may be disrupted when cellular zinc levels drop [Hesketh, 1982; Oteiza *et al.*, 1990]. Current drugs approved for use or in clinical trials to treat prostate cancer (paclitaxel, docetaxel, and 2-methoxyestradiol) target the microtubule network resulting in cell death [Lapidus *et al.*, 2004; Gilbert and Parker, 2005; Qadan *et al.*, 2001]. What is unknown is if zinc deficient individuals have alterations in the microtubule network such that the activity of these chemotherapeutic agents is altered. Therefore, we tested the hypothesis that inadequate amounts of zinc disrupts microtubule function and decreases efficacy of microtubule-targeting chemotherapeutic agents in prostate cancer cells.

BODY

AIM 1: Determine the effects of zinc deficiency on cellular physiology of cultured prostate cells. Because our cell culture model for zinc deficiency was novel, we added this aim in addition to the original aims proposed in the grant in order to adequately characterize this new system and enable us to rule out confounding effects (e.g. reduced cellular viability) from the effects on microtubule function tested below.

Accomplishments & Challenges:

1. Zinc deficiency culture conditions were established for prostate cancer cell lines LNCaP and PC3. These cell lines require media supplemented with 10% fetal bovine serum (FBS) for optimum growth. FBS is the predominant source of the zinc in culture media, so chelation strategies must be used to deplete the zinc (since getting serum from a severely zinc-depleted cow is unrealistic). Previous approaches have stripped zinc from FBS using dialysis against soluble chelators or extraction with immobilized chelators, typically DTPA or EDTA [Mackenzie *et al.*, 2002; Ho and Ames, 2002]. However, these chelators are not selective for zinc and cause depletion of many divalent metals; repletion of other metals is often incomplete. Therefore, we used a new immobilized chelator (TAEA) that was much more selective for zinc (*Figure 1*). Only copper decreased more than 20% and was thus added back after chelation. Zinc selectivity for TAEA has not been previously reported and will be fully described in our upcoming manuscript.
2. Zinc deficiency culture conditions did not cause significant loss of viability in prostate cancer cell lines LNCaP and PC3. Despite 50-60% reduction in extracellular zinc levels, cell lines did not demonstrate decreased viability as determined by trypan blue-based vital dye exclusion or tetrazolium dye reduction (*Figure 2*). The degree of zinc deficiency in this study was less severe than in previous studies, which is likely more physiologically relevant. Moreover, the chelation was likely more selective for zinc. Under these conditions, overall cell viability was not diminished.
3. Zinc deficiency culture conditions did not cause significant decreases in intracellular zinc content in prostate cancer cell lines LNCaP and PC3. After 3 months of exposure to culture conditions with different zinc levels for adequate adaptation, randomly cycling populations were analyzed for intracellular zinc content by inductively-coupled plasma spectrometry (*Figure 3*). Because the level of zinc deficiency we used was moderate, the cells were able to maintain intracellular zinc levels, presumably by increasing zinc intake and/or reducing zinc export. Others have reported reductions in intracellular zinc, but they used severe zinc deficiency and less-selective chelators [Mackenzie *et al.*, 2002; Ho and Ames, 2002].
4. Zinc deficiency culture conditions did not cause significant alterations in cell cycling kinetics in prostate cancer cell lines LNCaP and PC3. After 3 months of exposure to culture conditions with different zinc levels for adequate adaptation, randomly-cycling populations were analyzed for cell cycle kinetics by propidium iodide staining of DNA content quantitated by fluorescence-activated flow cytometry (*Figure 4*).

5. Zinc deficiency culture conditions have recently been established for prostate epithelial cells (PrEC). These cell lines require a proprietary serum-free media (Cambrex Bioproducts, Walkersville, MD) for optimum growth (no FBS). We had to establish an account with the company for custom preparation of PrEC media without any zinc added. This agreement and lead-time for preparation took approximately 6 months; custom media finally arrived in March 2006.

AIM 2: Determine the effects of zinc deficiency on microtubule structure and dynamics in cultured prostate cells. We realized upon starting this work that there are few previous studies on zinc's role in microtubule structure/function relationships. Therefore, we have added studies that characterize the effects of zinc availability in purified microtubules in addition to the microtubules from the cell lines. Biochemical work done using purified tubulin predicted 10 high affinity and 55 low affinity binding sites for zinc [Eagle *et al.*, 1983]. It has also been shown that low amounts of zinc can aid in assembly of microtubules [Gaskin, 1981]. Many investigators have proposed that zinc is important for a proper functioning microtubule network [Morisawa and Mori, 1972; Reinhold *et al.*, 1967], but the specific mechanisms and interactions are essentially unknown. After completing our studies on purified microtubules, we will be able to better understand the effects of zinc on the prostatic microtubule network. Different cell types can have strikingly different microtubule assemblies and activity due to differential expression of tubulin isoforms and/or microtubule-associating proteins. Therefore the purified microtubule studies will allow us to distinguish between an affect of zinc directly on microtubule protein versus other accessory factors that regulate the whole network.

Accomplishments & Challenges:

1. The ultrastructure of microtubules from purified tubulin protein stabilized with paclitaxel was not substantially altered when depleted of zinc using the zinc-selective chelator TPEN. Purified tubulin from bovine brain was assembled into microtubules *in vitro* in the presence of GTP and paclitaxel. Then, a dose-range of TPEN was applied to the preparation and imaged with electron microscopy at 16,000X power (*Figure 5*). Extraction of zinc with TPEN did not appear to cause any change in the microtubule structure or length. We are collecting additional data points for a more quantitative assessment. However, of greater interest is how microtubule structure and length will be affected when zinc is depleted before polymerizers are added. We are also working on this, including testing possible artifacts the effect of the chelator on other critical ions like magnesium.

2. The polymerization rate of microtubules from purified tubulin protein may be substantially altered in the presence of zinc or the zinc-selective chelator TPEN. In preliminary experiments, purified tubulin from bovine brain was allowed to assemble into microtubules *in vitro* in the presence of excess GTP while simultaneously measuring absorbance due to polymer formation on a spectrophotometer with a kinetic software package. Zinc dose-dependently favored polymerization rates, where all concentrations of TPEN blocked polymer formation (*Figure 6*). We are currently working on this to test for possible artifacts the effect of the chelator on other critical ions like magnesium. Once the kinetics of polymerization are defined, we will test the interaction of zinc on the activity of microtubule-polymerizing and microtubule-depolymerizing agents. Surprisingly, these seemingly basic studies on zinc and microtubule dynamic relationships have not been previously reported.

3. The structure of the microtubule network from prostate cancer cell lines LNCaP and PC3 does not appear to be altered by zinc deficient culture conditions. In preliminary experiments, the microtubule networks of prostate cells from control and zinc deficient conditions treated with or without paclitaxel were imaged using immunofluorescence microscopy (*Figure 7 & 8*). LNCaP and PC-3 cell lines were fixed in methanol after exposure to either control or zinc deficient culture conditions and stained with a tubulin antibody followed by a fluorescently labeled secondary antibody. As expected, 50 μ M paclitaxel treatment caused a marked increase in microtubule bundling. However, no qualitative differences in the microtubule networks – either in native structure or paclitaxel-treated - was evident as a function zinc status.

4. The polymerization rate of microtubules from prostate cancer cell lines LNCaP and PC3 may be altered in the presence of zinc or the zinc-selective chelator TPEN. We have developed an *in cyto* assay (modified from a commercial kit) to determine the ratio of tubulin heterodimers to microtubule polymers using differential ultracentrifugation and Western blot analysis. Details of this assay will be fully described in our upcoming manuscript. The experimental goal is to determine if the ratio of free tubulin to polymerized microtubules is altered within LNCaP and PC-3 cells exposed to normal or zinc deficient conditions. These studies are currently on-going.

AIM 3: Determine the effects of zinc deficiency-induced microtubule aberrations on chromatin packing and chromosome processing in cultured prostate cells. While microtubules have many functions within the cell, arguably of top importance is the organization of the nuclear material and proper separation of chromosomes with the spindle apparatus during mitosis/meiosis. This is of particular importance in the transition from pre-cancerous to cancerous phenotype, since disruptions in the microtubule network can promote genomic instability. Therefore, we also wanted to look at the way this immediate downstream target of microtubule structure/function may be altered during zinc deficient conditions.

Accomplishments & Challenges:

1. The chromatin/chromosome structure from prostate cancer cell lines LNCaP and PC3 does not appear to be altered by zinc deficient culture conditions alone. In preliminary experiments, the chromatin/chromosomes of prostate cells from control and zinc deficient conditions treated were imaged using immunofluorescence microscopy (*Figure 7 & 8*). Qualitative assessment did not indicate a substantially different pattern of the chromatin/chromosome structure under the conditions of zinc deficiency that we used. We continue to collect data on this in order to apply more quantitative assessment using significant numbers of cells in each category.

2. The chromatin/chromosome structure from prostate cancer cell lines LNCaP and PC3 did appear altered by zinc deficient culture conditions when exposed to paclitaxel. In preliminary experiments, the chromatin/chromosomes of prostate cells from control and zinc deficient conditions treated with or without paclitaxel were imaged using immunofluorescence microscopy (*Figure 7 & 8*). Paclitaxel treatment in cells from standard culture conditions resulted in greater than 75% of the cells having pinocytotic nuclei, indicating a progression towards apoptosis. Interestingly, paclitaxel treatment in cells from zinc deficient culture conditions only caused pinocytotic nuclei in approximately 50% of the cells. This suggests that zinc deficiency may affect the indirect changes in chromatin/chromosome processing caused by microtubule stabilizing drugs.

3. Chromosome processing through the cell cycle from prostate cancer cell lines LNCaP and PC3 does not appear to be altered by zinc deficient culture conditions alone. Randomly cycling populations under standard or zinc deficient culture conditions were analyzed for cell cycle distribution by propidium iodide staining of DNA content and quantification by fluorescence-activated flow cytometry (*Figure 4*). There was no significant difference in cell cycling kinetics in either cell type as a function of zinc status.

AIM 4: Determine the effects of zinc deficiency on efficacy of chemotherapeutic agents that target microtubules in cultured prostate cells. This part of the project is most translational, since it studies the interaction between zinc status and chemotherapeutic efficacy. We were able to show an exciting result, i.e. that a drug used against prostate cancer (paclitaxel) had reduced efficacy when prostate cancer cells were maintained under moderately zinc deficient conditions. These drugs have very small therapeutic windows, thus reduced efficacy might require greater dosing and increased side-effects. Moreover, the reduction in efficacy may also promote more rapid acclimatization and resistance to the drug class, leading to the development of more refractory cancer phenotypes. Our findings now should be extended to animal models for necessary vertical integration.

Accomplishments & Challenges:

1. Paclitaxel efficacy as a function of zinc status was determined in prostate cancer cell lines LNCaP and PC3 by analyzing changes in cell cycling kinetics. After adaptation period to culture conditions with different zinc levels, randomly-cycling populations were exposed to a dose-range of paclitaxel for 24 hours and then analyzed for cell cycle kinetics by propidium iodide staining of DNA content quantitated by fluorescence-activated flow cytometry (*Figure 9*). In zinc deficient LNCaPs, paclitaxel demonstrated decreased efficacy, with a 10-fold increase in IC50 compared to control and zinc repleted cells. In PC3s, paclitaxel demonstrated similar efficacy in all groups. So in the androgen-sensitive prostate LNCaP line alone, paclitaxel had reduced activity when cells were maintained under zinc deficient conditions.

2. Docetaxel efficacy as a function of zinc status was determined in prostate cancer cell lines LNCaP and PC3 by analyzing changes in cell cycling kinetics. After adaptation period to culture conditions with different zinc levels, randomly-cycling populations were exposed to a dose-range of docetaxel for 24 hours and then analyzed for cell cycle kinetics by propidium iodide staining of DNA content quantitated by fluorescence-activated flow cytometry

(data not shown). In three different experiments, docetaxel-treated LNCaPs have demonstrated three completely different efficacy patterns when comparing cells from standard, zinc deficient, and zinc repleted media. We are presently attempting to define the uncontrolled variables that might be influencing our LNCaP results. However, docetaxel-treated PC3s have demonstrated a uniform response identical to the paclitaxel response, namely equal efficacies regardless of whether cells were from zinc adequate or deficient cultures.

3. 2-Methoxyestradiol (2ME) efficacy as a function of zinc status was determined in prostate cancer cell lines LNCaP and PC3 by analyzing changes in cell cycling kinetics. After adaptation period to culture conditions with different zinc levels, randomly-cycling populations were exposed to a dose-range of 2ME for 24 hours and then analyzed for cell cycle kinetics by propidium iodide staining of DNA content quantitated by fluorescence-activated flow cytometry (*data not shown*). In preliminary experiments, 2ME was equally efficacious regardless of whether cells were from zinc adequate or deficient cultures in both LNCaP and PC3 cell types. Unlike paclitaxel and docetaxel, 2ME is an inhibitor of microtubule polymerization that interacts with the tubulin protein via a unique binding site [Downing, 2000]. It may not be surprising then that 2ME would respond differently than the taxane drug class to changes in microtubule structure/function relationships - a proposed effect of zinc deficiency. Current research has suggested that using 2ME in conjunction with paclitaxel may have a synergistic effect on apoptosis of the cancer cell since 2ME suppresses transcripts and protein from an isotype of tubulin that is overexpressed in paclitaxel resistance [Montgomery *et al.*, 2005].

4. Paclitaxel-stimulated apoptosis as a function of zinc status was determined in prostate cancer cell lines LNCaP and PC3 by analyzing (1) sub-G1 DNA content, (2) annexin staining, and (3) fluorescence microscopy.

- (1) For DNA content, randomly-cycling populations were exposed to a dose-range of paclitaxel for 24 hours and then analyzed for cell cycle kinetics by propidium iodide staining of DNA content followed by quantitation using fluorescence-activated flow cytometry (*Figure 10*). In zinc deficient LNCaPs, paclitaxel treatment caused reduced number of cells with sub-G1 DNA content at the IC₅₀ range of the drug as compared to control and zinc repleted cells. In PC3s, paclitaxel demonstrated similar efficacy in all groups. Thus, paclitaxel had reduced activity in cells maintained under zinc deficient conditions, though only in the androgen-sensitive prostate LNCaP line.
- (2) For annexin staining, randomly cycling populations were exposed to a dose-range of paclitaxel for 24 hours and then analyzed for annexin positivity by immunocytochemistry followed by quantitation using fluorescence-activated flow cytometry (*data not shown*). Unfortunately, LNCaP cells were intolerant of the buffer conditions required for immunocytochemistry, resulting in clumped cells that could not be used by flow cytometry. In PC3s (which tolerated buffer conditions), paclitaxel treatment caused similar degrees of annexin positivity in all groups, regardless of whether cells were from zinc adequate or deficient cultures.
- (3) For fluorescence microscopy, randomly-cycling populations were exposed to a dose-range of paclitaxel for 24 hours and then the nuclear chromatin was imaged by DAPI staining followed by analysis using fluorescence microscopy (*Figure 7 & 8*). In zinc deficient LNCaPs, paclitaxel treatment caused reduced number of cells with pinocytotic nuclei (a hallmark of apoptosis) as compared to control and zinc repleted cells at the IC₅₀ range of the drug. In PC3s, paclitaxel at the same dose demonstrated similar efficacy in all groups. So again, paclitaxel had reduced activity in cells maintained under zinc deficient conditions, though only in the androgen-sensitive prostate LNCaP line.

5. Docetaxel-stimulated apoptosis as a function of zinc status was/will be determined in prostate cancer cell lines LNCaP and PC3 by analyzing sub-G1 DNA content, (2) annexin staining, and (3) fluorescence microscopy.

- (1) For DNA content, randomly-cycling populations were exposed to a dose-range of docetaxel for 24 hours and then analyzed for cell cycle kinetics by propidium iodide staining of DNA content quantitated by fluorescence-activated flow cytometry (*data not shown*). In three different experiments with zinc deficient LNCaPs, docetaxel had differing effects on cell number with sub-G1 DNA content such that a predictable pattern has not yet emerged. We are attempting to define the uncontrolled variables that might be influencing the docetaxel effect on LNCaP apoptotic markers. However in PC3s, docetaxel demonstrated reproducible and similar efficacy in all groups, similar to paclitaxel.
- (2) For annexin staining, we will start these experiments once the buffer conditions that are compatible for the use of immunocytochemistry with LNCaPs and subsequent flow cytometry are determined.
- (3) For fluorescence microscopy, we have prepared the cell samples and are waiting for microscope time to image the cells.

6. 2ME-stimulated apoptosis as a function of zinc status was/will be determined in prostate cancer cell lines LNCaP and PC3 by analyzing (1) sub-G1 DNA content and (2) annexin staining, and (3) fluorescence microscopy.

- (1) For DNA content, randomly-cycling populations were exposed to a dose-range of 2ME for 24 hours and then analyzed for cell cycle kinetics by propidium iodide staining of DNA content quantitated by fluorescence-activated flow cytometry (*data not shown*). In three different experiments with zinc deficient LNCaPs, 2ME demonstrated reproducible and similar efficacy in all groups in both LNCaP and PC3 cell lines, similar to its effect on cell cycling kinetics.
- (2) For annexin staining, we will start these experiments once the buffer conditions that are compatible for the use of immunocytochemistry with LNCaPs and subsequent flow cytometry are determined.
- (3) For fluorescence microscopy, we have prepared the cell samples and are waiting for microscope time to image the cells.

Current Activities:

1. Establish zinc deficiency culture conditions with primary prostate epithelial cells (PrEC) on cell physiology and microtubule function with recently acquired custom media. Chelation of zinc within the media is unnecessary because no FBS is used; PrEC media is completely synthetic. Zinc deficient media was created by custom synthesis as described above.

2. Determine rates of zinc uptake in prostate cell lines maintained during zinc deficiency culture conditions using isotopic zinc tracers and subsequent detection by inductively-coupled plasma spectrometry/mass spectrometry. A colleague at our institution uses stable zinc isotopes for similar measurements in animals and is willing to consult on this study. Increased uptake rates in zinc deficiency culture conditions would explain how intracellular zinc levels are kept constant.

3. We have proposed to measure microtubule dynamics in live prostate cells under normal and zinc deficient culture conditions using time-lapse fluorescence microscopy. To do this, we purchased rhodamine-labeled tubulin that will be microinjected into the cells to integrate into the endogenous tubulin pool for subsequent kinetic analysis in live cells. However, we later realized that the microinjection system we had planned to use is not suitable for our experimental design. Therefore, we are investigating the technical capacities of other microinjection systems on campus that could be used for our analyses. We are especially interested in seeing how paclitaxel and the other microtubule-targeting drugs will affect the *in cyto* dynamics as a function of zinc status.

4. LNCaPs proved difficult to analyze for annexin positivity as a marker of apoptosis as they were intolerant of the required buffers for immunocytochemistry and subsequent flow cytometry. Manipulation of the cells in these buffers quickly resulted in cell clumps that could not be mechanically broken up and thus prevented analysis by cytometry. Reducing extracellular calcium levels has been used to attenuate cell clumping in other assays, but the calcium levels in the buffer are required for antibody-annexin interaction. We are in the process of trying additional protocols, including adding albumin or heparin to prevent cell sticking. However, other methods were also used as described above to quantitate apoptosis in this cell line.

5. We have prepared lysates from LNCaPs and PCs at multiple timepoints for Western blot analysis in order to determine if zinc deficient conditions cause changes in tubulin protein levels. Tubulin content has been measured in rat models of zinc deficiency with no reported change between control and deficient conditions [Oteiza *et al.*, 1990]. However, microtubule polymerization rates were decreased in zinc deficient rats in that study. The authors suggested that zinc deficiency might cause a change in the expression of tubulin isotypes. This would be of additional interest because paclitaxel has different binding affinities with the different tubulin isotypes. We are pursuing the use of isotype-specific antibodies to test this possibility.

KEY RESEARCH ACCOMPLISHMENTS:

- Established novel zinc deficiency culture conditions using zinc-selective extraction.
- Established a zinc deficiency culture model for transformed human prostate cell lines (LNCaP and PC-3).
- With moderate zinc deficiency conditions, no significant loss of LNCaP or PC-3 viability was detected.
- With moderate zinc deficiency conditions, LNCaP or PC-3 maintained intracellular zinc levels.
- Zinc deficiency did not appear to alter microtubule structure as determined by electron microscopy.
- Zinc deficiency did appear to alter microtubule dynamics as determined by polymerization studies.

- Zinc deficiency did not alter chromatin structure as determined by fluorescence microscopy.
- Zinc deficiency altered chromatin processing when in the presence of paclitaxel as determined by fluorescence microscopy.
- Zinc deficiency reduced paclitaxel-mediated cell cycle arrest in LNCaPs, but not PC3s.
- Zinc deficiency reduced paclitaxel-mediated apoptosis in LNCaPs, but not PC3s.
- Studies with other microtubule-targeting chemotherapeutics and zinc status are underway.

REPORTABLE OUTCOMES:

- Manuscript detailing reduced paclitaxel efficacy during zinc deficiency – in preparation
- Presentations of work describing reduced paclitaxel efficacy during zinc deficiency to CHRCO Cancer Center and during 2006 CHORI Research Symposium – pending
- Collaboration with Dr. Emily Ho (Oregon State University) to evaluate combination therapy of natural HDAC inhibitors with new microtubule-targeting drugs
- Results have generated enough interest to merit inclusion of project goals into Ph.D. thesis goal for lead graduate student working on this proposal

CONCLUSIONS:

We have established a novel cell culture model for zinc deficiency using a more zinc selective chelator. Zinc deficiency was more moderate, and thus more physiological, than previously described studies; moreover, cells were exposed for many months to these new zinc levels to allow them to fully acclimate. Our characterization studies showed that the prostate cancer cell lines LNCaPs and PC3s tolerated these culture conditions well with no obvious lack of viability, reducing possible confounders from the effects zinc deficiency on microtubule function we tested later. Because there are few studies reported on the role of zinc in microtubule structure/function relationships, we added several studies to our original research goals to begin characterizing the effects of zinc on purified microtubules in addition to the microtubules from the cell lines. We found that zinc levels did affect dynamics in isolated microtubules. The dynamic nature is critical for normal cellular division and thus a major target of anti-cancer therapeutics. These findings now need to be assessed in cells, as different cell types can have radically different microtubule assemblies due to differential expression of tubulin isoforms and/or microtubule-associating proteins. These studies are underway, but preliminary evidence suggests that microtubule dynamics may be altered by zinc status in (prostate) cells as well. Additionally, chromatin processing, an immediate downstream target of microtubule activity, may also be altered during zinc deficient conditions. The most translational part of the study is the interaction between zinc status and chemotherapeutic efficacy. We found that a drug used against prostate cancer (paclitaxel) had reduced efficacy in causing cell cycle arrest and apoptosis when prostate cancer cells were maintained under moderately zinc deficient conditions. These findings should now be tested to animal models to see if the effect translates to whole animals while concomitant molecular studies should determine the mechanisms. Since zinc deficiency is common in the US, this nutritional status may be of importance when creating a treatment strategy for prostate cancer. Reduced efficacy of these drugs might exaggerate side-effects and promote resistance within the cancer.

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APPENDIX: Figures to follow.

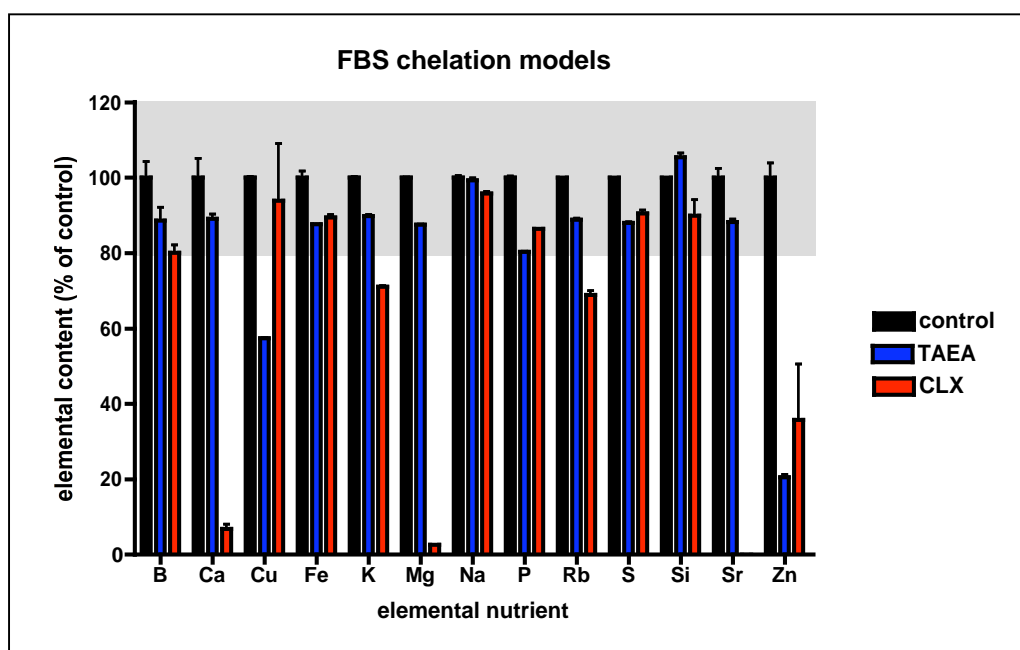


Figure 1: TAEA immobilized chelator beads are superior to CHELEX-100 (CLX) chelator beads for zinc selectivity. Representative data shows mean \pm SD from elemental analysis of fetal bovine serum treated with TAEA or CLX beads at 10% (w/v) for 10 minutes at 4°C with constant inversion. Triplicate aliquots of serum before (control) and after chelator extraction were analyzed for elemental content using ICP-AES and expressed as percent of control.

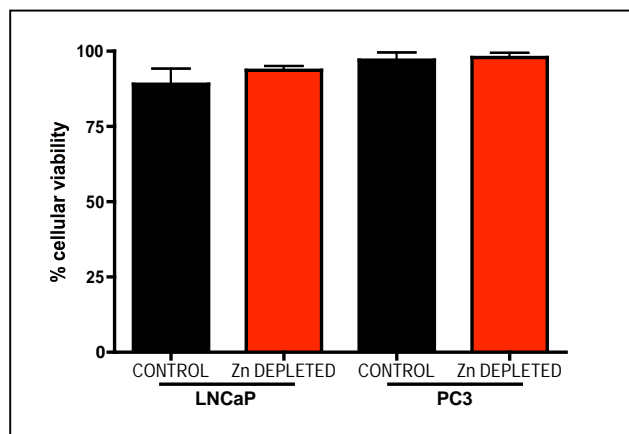


Figure 2: Moderate zinc deficiency does not reduce cellular viability in prostate cell lines. Representative data shows mean \pm SD from viability assay of LNCaPs and PC3s after 3 months acclimation to standard or zinc deficient culture conditions. Triplicate wells of subconfluent LNCaPs and PC3s were incubated for 5 minutes with 0.2% trypan blue and then scored for dye exclusion by light microscopy. Cellular viability was reduced dose-dependently by H_2O_2 treatment as a positive control (data not shown). These results were also similar to viability as detected by tetrazolium reduction assays (data not shown).

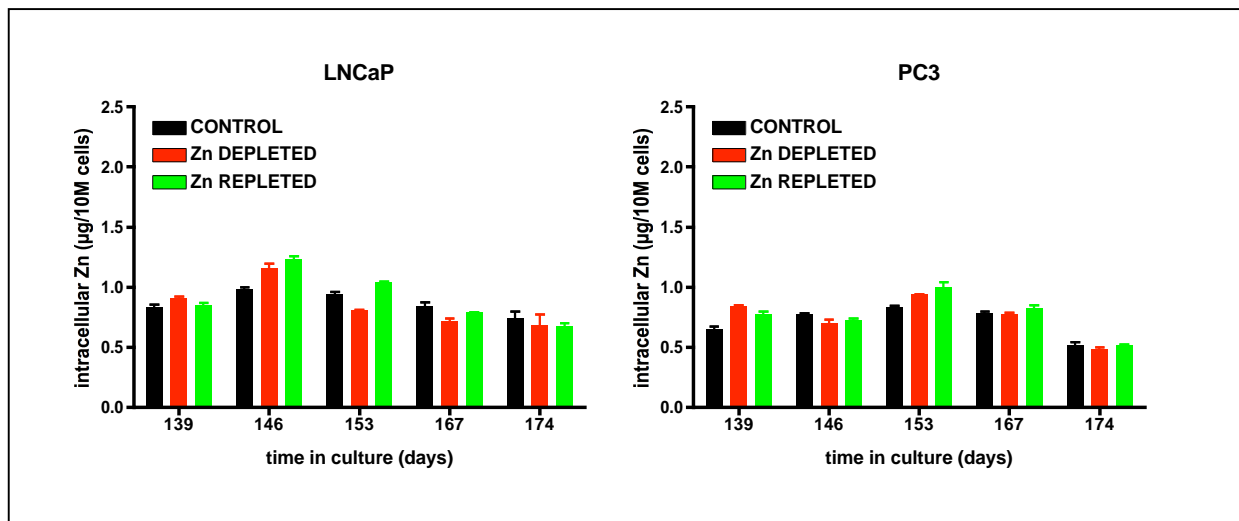


Figure 3: Intracellular zinc levels in prostate cell lines are not significantly decreased during moderate zinc deficiency culture conditions. Representative data shows mean \pm SD from elemental analysis of LNCaPs and PC3s after increasing time in standard, zinc deficient, or zinc repleted media. Duplicate pellets of 10 million (10M) cells were analyzed for elemental content using ICP-AES and normalized to cell number.

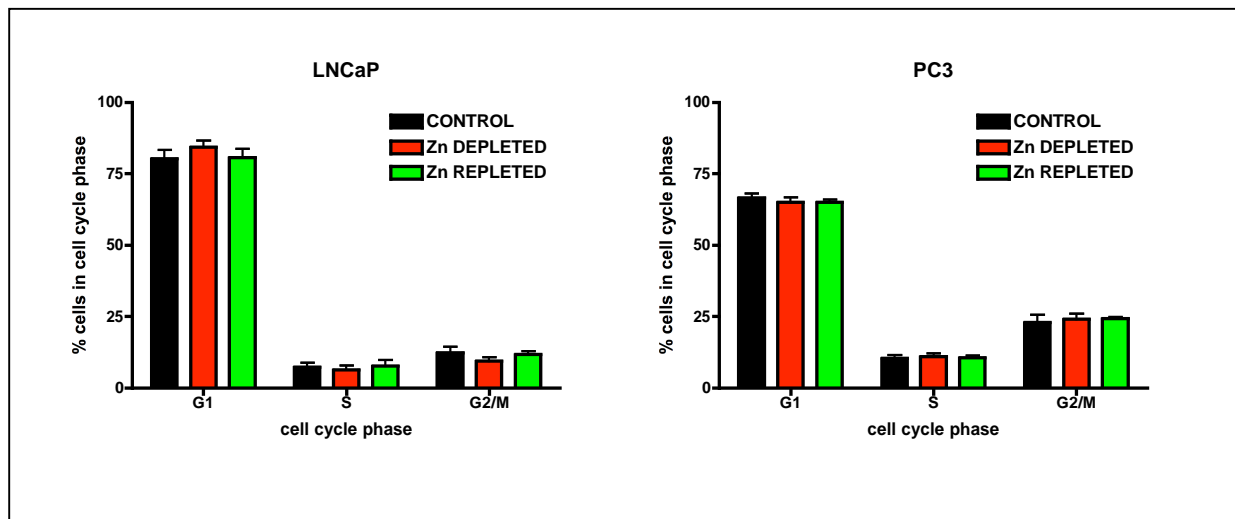


Figure 4: Moderate zinc deficiency does not alter cell cycle kinetics in prostate cell lines. Representative data shows mean \pm SD from quantitation of the DNA content of LNCaP and PC3 cells after 3-4 months acclimation to standard or zinc deficient culture conditions. Three independent, subconfluent, randomly cycling LNCaP and PC3 cells were treated with propidium iodide and then analyzed for DNA content by flow cytometry. Cell cycle distribution was determined using flow cytometry native software (CellQuest; Becton Dickinson).

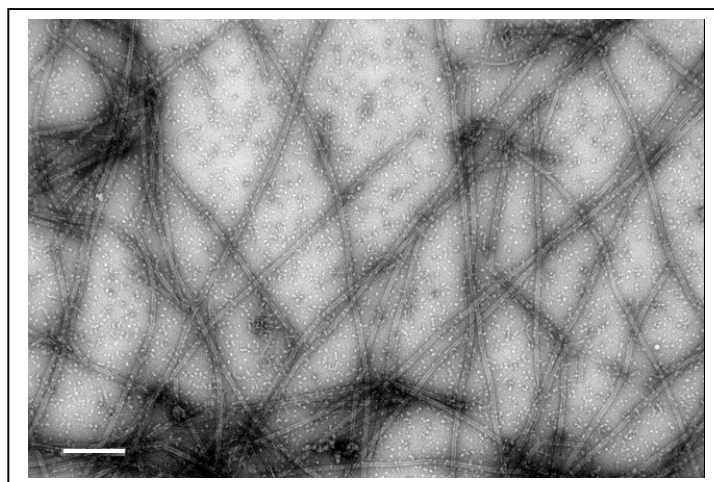


Figure 5: Zinc depletion does not qualitatively alter microtubule ultrastructure. Representative image shows microtubule assemblies from purified bovine tubulin protein stabilized with paclitaxel and imaged by electron microscopy at 16,000X power. Scale bar indicates 0.5 μm . Treatment of microtubule assemblies with a dose range of the zinc-selective chelator TPEN did not cause detectable changes in microtubule structure (data not shown). Alterations in the content and exposure time of tubulin, zinc, TPEN, paclitaxel and other parameters are in progress.

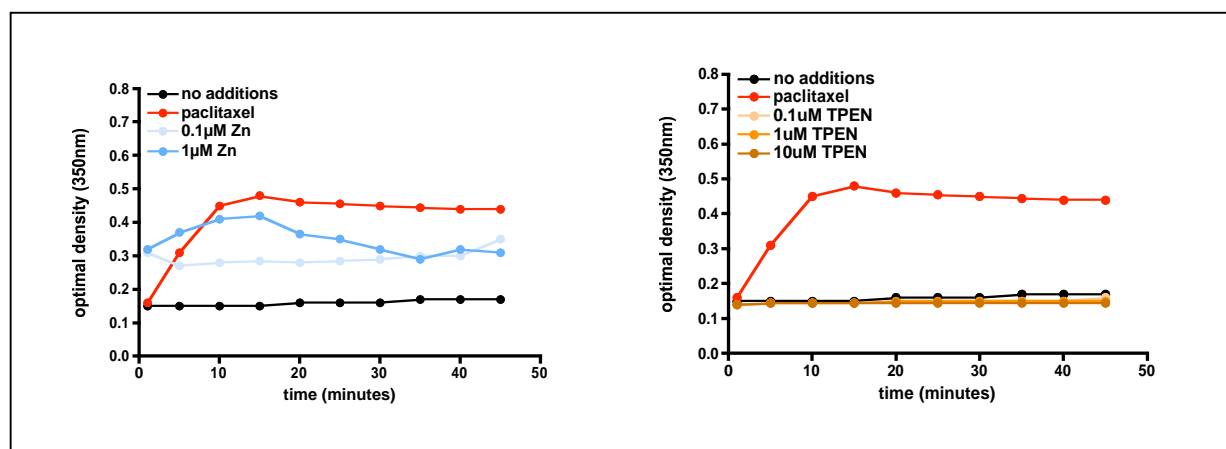


Figure 6: Zinc availability positively affects microtubule polymerization rates. Preliminary experiment shows microtubule assembly rates of purified bovine tubulin protein stabilized with treatments of zinc, TPEN, and/or paclitaxel. Separate treatment conditions were prepared on ice and then warmed to 37°C with simultaneous measurement of optical density (350 nm) over the span of 1 hour. Free zinc (0.1-10 μM) promoted, while TPEN (0.1-10 μM) inhibited, microtubule polymerization. Alterations in the content and exposure time of tubulin, zinc, TPEN, paclitaxel and other parameters are in progress.

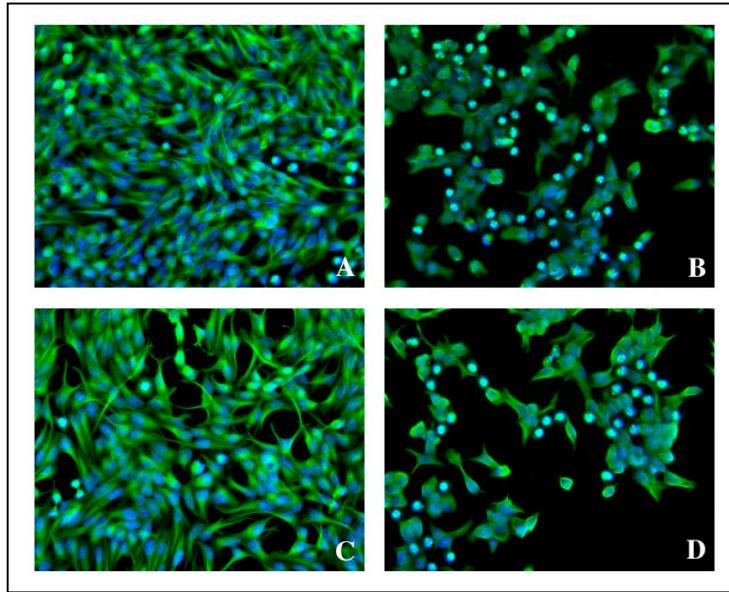


Figure 7: Moderate zinc deficiency does not alter microtubule networks but reduces apoptotic nuclei in LNCaPs. Representative image shows microtubules (green) and nuclei (blue) from LNCaPs grown in standard or zinc deficient culture conditions treated with or without 50 nM paclitaxel. Fixed cells were incubated with primary mouse-anti-bovine tubulin and AlexaFluor-488-labeled secondary goat-anti-mouse IgG antibodies, and imaged with fluorescence microscopy (160X). Panels are (A) LNCaPs in standard media - paclitaxel, (B) LNCaPs in standard media + paclitaxel, (C) LNCaPs in zinc deficient media – paclitaxel, and (D) LNCaPs in zinc deficient media + paclitaxel. Microtubule networks are similar but there are less apoptotic nuclei in paclitaxel-treated cells from zinc deficient media compared to standard media.

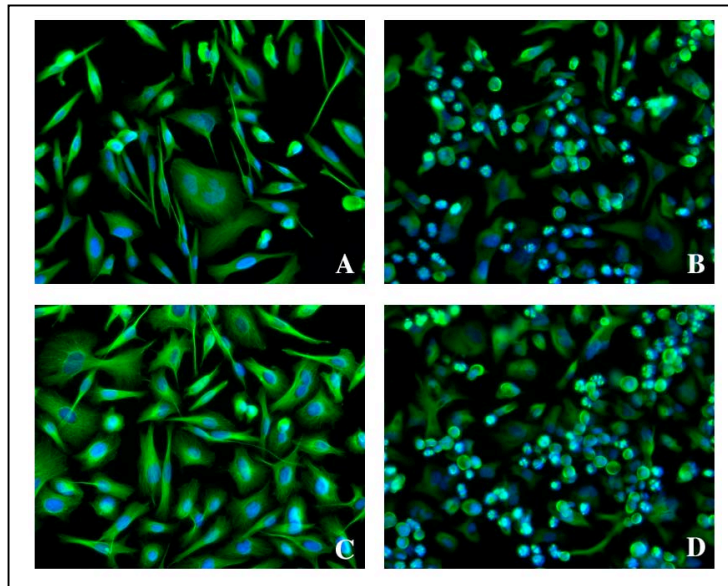


Figure 8: Moderate zinc deficiency does not alter microtubule networks or apoptotic nuclei in PC3s. Representative image shows microtubules (green) and nuclei (blue) from PC3s grown in standard or zinc deficient culture conditions treated with or without 50 nM paclitaxel. Fixed cells were incubated with primary mouse-anti-bovine tubulin and AlexaFluor-488-labeled secondary goat-anti-mouse IgG antibodies, and then imaged with fluorescence microscopy (160X). Panels are (A) PC3 in standard media - paclitaxel, (B) PC3 in standard media + paclitaxel, (C) PC3 in zinc deficient media – paclitaxel, and (D) PC3 in zinc deficient media + paclitaxel. Microtubule networks and apoptotic nuclei numbers are similar in paclitaxel-treated cells from standard or zinc deficient media.

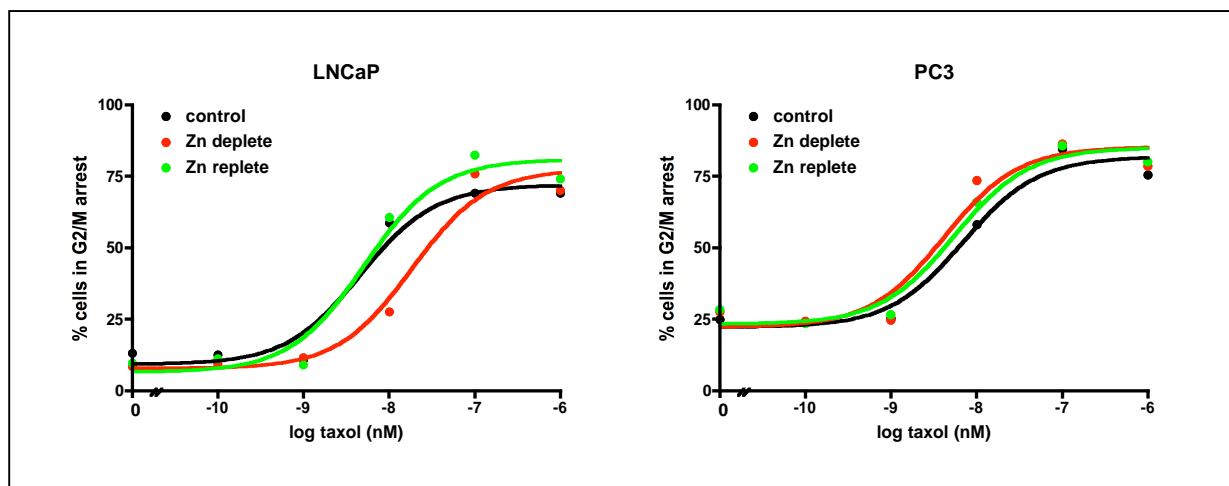


Figure 9: Moderate zinc deficiency reduced paclitaxel efficacy in prostate cell lines. Representative data shows mean \pm SD from quantitation of the DNA content of LNCaP and PC3 cells after 3-4 months acclimation to standard or zinc deficient culture conditions. Three independent, subconfluent, randomly cycling LNCaP and PC3 cells were treated with propidium iodide and then analyzed for DNA content by flow cytometry. Cell cycle distribution was determined using flow cytometry native software (CellQuest; Becton Dickinson).

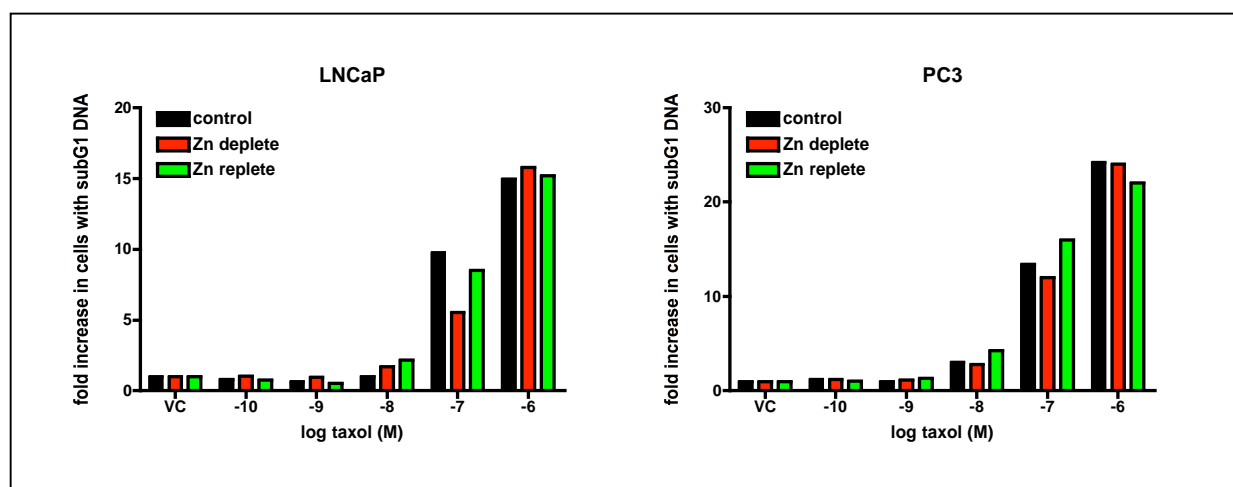


Figure 10: Moderate zinc deficiency reduced paclitaxel-induced apoptosis in prostate cell lines in the IC50 range. Representative data shows mean \pm SD from quantitation of the sub-G1 DNA content of LNCaP and PC3 cells after 3-4 months acclimation to standard or zinc deficient culture conditions. Randomly cycling LNCaP and PC3 cells were treated with propidium iodide and then analyzed for DNA content by flow cytometry. Sub-G1 cell cycle distribution was determined using flow cytometry native software (CellQuest; Becton Dickinson).